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Review

Neutrophil extracellular traps (NETs) and fibrotic diseases

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ABSTRACT

Fibrosis, a common cause and serious outcome of organ failure that can affect any organ, is responsible for up to 45% of all deaths in various clinical settings. Both preclinical models and clinical trials investigating various organ systems have shown that fibrosis is a highly dynamic process. Although many studies have sought to gain understanding of the mechanism of fibrosis progression, their findings have been mixed. In recent years, increasing evidence indicates that neutrophil extracellular traps (NETs) are involved in many inflammatory and autoimmune disorders and participate in the regulation of fibrotic processes in various organs and systems. In this review, we summarize the current understanding of the role of NETs in fibrosis development and progression and their possibility as therapeutic targets.

1. Introduction

Fibrosis is the result of dysregulated tissue repair response in the course of various types of tissue injury, especially that incurred by chronic inflammatory diseases [1]. Activation of fibroblasts, secretion of inflammatory mediators, and synthesis of extracellular matrix (ECM) are increased in fibrotic tissues and organs, leading to the disruption of physiological architecture and organ dysfunction [2]. As the global incidence of fibrosis and the accompanying healthcare burden continues to increase, fibrosis is becoming more widely acknowledged as a significant healthcare concern [3,4]. There is mounting evidence that many molecular mechanisms play a key role in the process of fibrosis [1], including functional heterogeneity and fibroblast plasticity. Studies have shown that activation and inactivation of the transcription factor PU.1 leads to hyperactivity and immobilization, respectively, of fibroblast function [5]. In transforming growth factor beta 1 (TGFβ1)-stimulated fibroblasts, the conversion of glutamine to glutamate under the action of glutaminase is enhanced [6], which increases fibroblast resistance to apoptosis [7] and promotes the stabilization of collagen through mammalian target of rapamycin (mTOR) signaling [6].

The absence of certain macrophage subpopulations can aggravate fibrosis [8,9]. However, macrophage-derived amphiregulin can encourage mesenchymal stromal cells to develop into myofibroblasts via integrin-αV-mediated activation of TGFβ, resulting in pathological ECM deposition [10]. The αvβ1, αvβ3, αvβ5, αvβ6, and αvβ8 integrins have all been demonstrated to activate TGFβ [11–13]. In addition to TGF, a number of other cytokines, including members of the interleukin (IL)-17 family [14–19] and the type 2-associated cytokines IL-4 and IL-13 [20–23], are implicated in fibrosis induction. There is also a strong link between the microbiome and the development of fibrosis. In an experimental model of liver fibrosis, Seki et al. observed alleviation of fibrosis by reduction of hepatic exposure to intestinal microbes or blocking of toll-like receptor 4 (TLR4) signaling [24].

Mainly composed of DNA, histones, and granule proteins, neutrophil extracellular traps (NETs) are web-like structures released by activated neutrophils [25]. Pathogens such as bacteria [26], fungi [27], parasites, and viruses [28,29], activated platelets [30], certain chemokines, phorbol myristate acetate (PMA), and Ionomycin [31] all contribute to NET formation and release. Referred to as NETosis, NET formation is a cell-death program unique form of cell death that is characterized by release of decondensed chromatin and granular contents to the extracellular space distinct from that of apoptosis and necrosis. The current mainstream view divides the sources of NETs into nuclear and mitochondrial sources, of which NETs formed from the former are classified as lytic and nonlytic by Kubes et al. [32]. After stimulation, neutrophils undergo cell depolarization, chromatin depolymerization, nuclear membrane lysis, cell membrane rupture, and NET release [33], in which reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity, reactive oxygen species (ROS) formation, and activation of peptide arginine deiminase 4 (PAD4) are key events [34–36]. The characteristic feature of nonlytic NET release is the release of DNA and proteins through exocytosis of neutrophils, which allows the neutrophils

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to retain some function, rather than through rupture of the cell membranes of neutrophils [37–39] (Fig. 1).

As mitochondrial NETosis has only recently been identified as part of the NET production pathway, its specific mechanisms require further elucidation [40,41]. Although NETs can capture and kill various pathogens, they can also cause tissue damage under certain circumstances. An increasing number of studies have reported that NETs are capable of inducing fibrotic disorders following certain types of tissue injury (Fig. 2). In this review, we summarize the current knowledge of NETs in fibrotic diseases in various human systems.

2. The roles of NETs in human fibrotic diseases

2.1. The cardiovascular system

Remaining a treatment challenge in the field of cardiology, cardiac fibrosis is characterized by the activation of cardiac fibroblasts after initial injury and their subsequent differentiation into myofibroblasts, the primary mediator cells in the pathological remodeling process. The persistent functions of myofibroblasts, including fibroblast proliferation and secretion, ECM turnover enhancement, and collagen deposition, result in fibrotic scarring and cardiac dysfunction[42].

Recent studies have highlighted the potential link between NET formation and cardiac fibrosis. In animal models of myocardial infarction (MI), inflammatory infiltration of neutrophils has been observed in heart tissue. In a study of histidine decarboxylase (HDC)-deficient mice, disordered recruitment of neutrophils was observed in the ischemia region of the myocardium, as well as promotion of greater cardiomyocyte death and cardiac fibroblast proliferation in $HDC^{-/-}$-mouse-derived neutrophils than wild-type (WT)-mouse-derived neutrophils [43]. Deoxyribonuclease (DNase) I treatment can blur the line between cardiomyocyte death and cardiac fibroblast proliferation, suggesting that $HDC^{-/-}$-mouse-derived neutrophils promote cardiomyocyte death as well as fibroblast proliferation and migration via NETosis. After observing that protein arginine methyltransferase 1 (PRMT1) upregulation mediated enhanced ROS production and NETosis in the neutrophils of $HDC^{-/-}$ mice, researchers concluded that the HDC-PRMT1-ROS-NET pathway is responsible for increased myocardial damage [43].

After an MI event, inflammatory stimuli, such as IL-17 and TNF-α, downregulate the expression of epidermal growth factor receptor (EGF)-like repeats and discoidin domains 3 (EDIL3). In an MI mouse model with permanent left anterior descending coronary artery (LAD) ligation, Wei et al. observed that EDIL3 deficiency boosts neutrophil recruitment and NET production [44]. These processes in turn drive the TLR9 pathway to promote the polarization of myeloid-epithelial-reproductive tyrosine kinase/major histocompatibility complex II ($Mertk^{-}MHC\text{-}II^{lo\text{-}int}$) macrophages. These pro-inflammatory macrophages improve adverse cardiac healing by inhibiting excessive collagen deposition through matrix degradation and eliminating necrotic cardiomyocyte debris through proteolysis and phagocytosis. Wei et al. attributed myocardial damage in an ischemia/reperfusion (I/R) model to NET-induced coronary thrombosis and attributed enhancement of cardiac function in the absence of thrombosis to NET-mediated pro-inflammatory $Mertk^{-}MHC\text{-}II^{lo\text{-}int}$ macrophage polarization in the I/R model.

Persistent fibrosis after MI eventually leads to the formation of ventricular aneurysm (VA), which is characterized by extensive collagen deposition and substantial myocardium loss [45]. Damage-associated molecular patterns (DAMP) secretion induced by MI initiates NETosis and NET secretion, which have been identified in the VA of both rats and humans. The accumulation of NETs compromises endothelium integrity and penetrates the myocardium via the TLRs-nuclear factor-kappa B (NF-κB) signaling pathway to activate cardiac fibroblasts. Subsequently, the cells secrete TGFβ and other pro-fibrotic effectors, activating the suppressor of mothers against decapentaplegic (Smad), mitogen-activated protein kinase (MAPK), and Ras homolog family member A (RhoA) signaling pathways and further exacerbating cardiac fibrosis.

In addition to fibroblasts, fibrocytes, a type of circulating cell possessing characteristics of leukocytes and fibroblasts [46], have recently been implicated in the fibrotic process. In patients with ST-segment elevation myocardial infarction (STEMI), neutrophils generate NETs at the culprit site, which stimulates the development of monocytes into fibrocytes. Activated by NETs, fibrocytes express cluster of differentiation molecule 11B (CD11B), adhere to endothelial cells via contact with intercellular adhesion molecule 1 (ICAM-1), and move into the myocardium, causing undesirable remodeling [47]. (Fig. 3). Research has demonstrated that the myocardium of mice with heart failure with

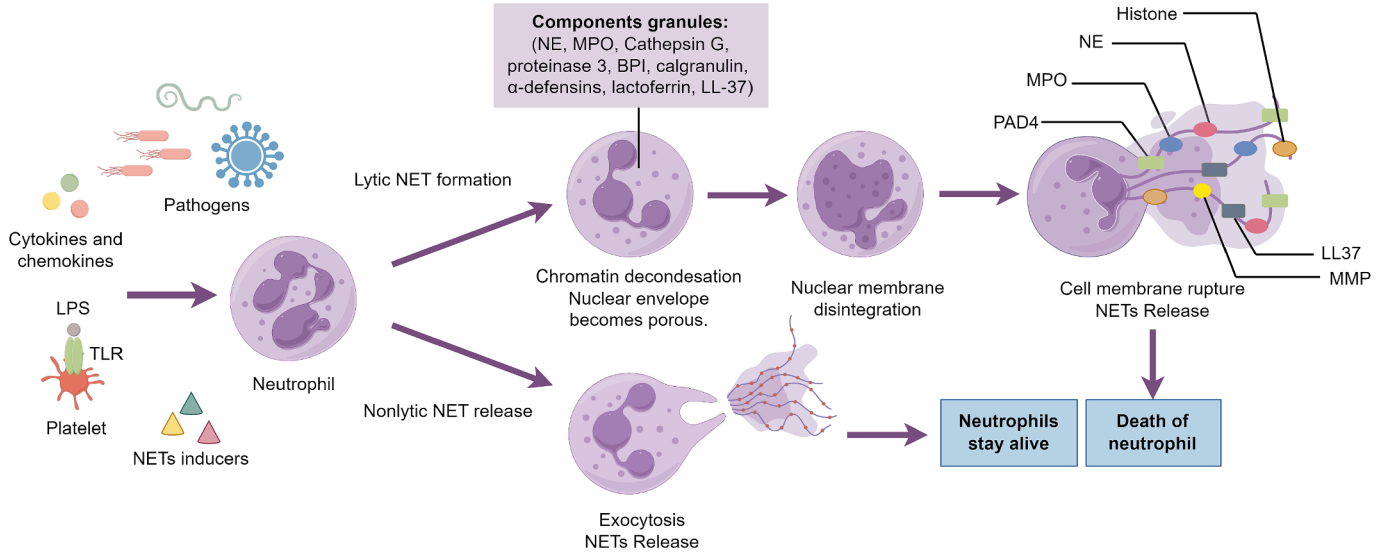

Fig. 1. The formation of NETs from the nucleus. Under stimulation, the NADPH oxidase (NOX) complex in neutrophils undergoes phosphorylation and releases reactive oxygen species (ROS), leading to the activation of peptide arginine deiminase 4 (PAD4) and facilitating the transfer of neutrophil elastase (NE) and myeloperoxidase (MPO) to the nucleus. Lytic NETs are released after chromatin depolymerization, nuclear membrane rupture, cell membrane rupture, etc, resulting in neutrophil death due to membrane disintegration. However, the formation of nonlytic NETs does not undergo nuclear or cell membrane rupture, but is released through exocytosis, while associated neutrophils retain certain functionality. NETs are mainly composed of DNA, histone and granule proteins. LL37, antibacterial protein ll-37 amide; MMP, matrix metalloproteinase. By Figdraw.

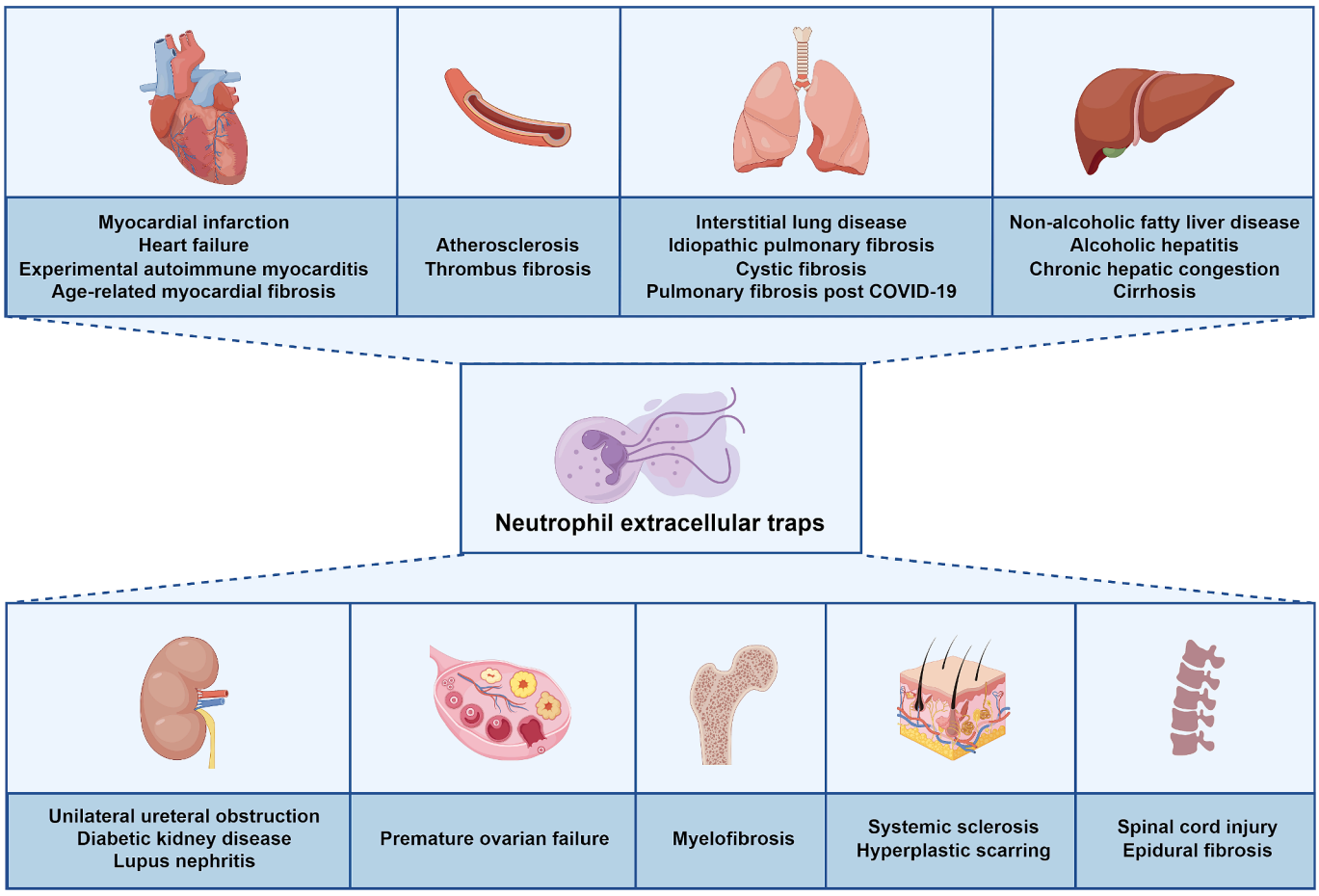

Fig. 2. Fibrotic diseases associated with NETs. By Figdraw.

preserved ejection fraction (HFpEF) has a high level of neutrophil infiltration and NET formation. In seipin knockout (SKO) mice, a murine model that develops HFpEF, Bai et al. found that NETs were linked to phosphorylation of titin and elevated extracellular interstitial fibrosis, which may cause left ventricular (LV) stiffness. Furthermore, they observed that the heart tissue of seipin / Bslc2 knockout mice showed hyperphosphorylation of Janus kinase 2 (JAK2) and its downstream signal transducer and activator of transcription 3 (STAT3), indicating a potential association between JAK2 signaling and NETosis and myocardial dysfunction [48].

High-mobility group protein B1 (HMGB1) has been demonstrated in various investigations to recruit and activate neutrophils [49], stimulate NETs production [50], and accelerate organ fibrosis [49,51,52]. In a murine model of heart failure with preserved ejection fraction (HFpEF), Zhang et al. observed that HMGB1 was elevated in heart tissue but was downregulated by treatment with sodium-glucose cotransporter-2 (SGLT2) inhibitor, which reduces cardiac neutrophil infiltration and NETs formation and improves diastolic function [53].

Martinod et al. found that aged mice had a higher incidence of NETosis, which depends on protein arginine deiminase 4 (PAD4) expression and can lead to impaired cardiac function [54]. Compared to WT mice, aged PAD4$^{-/-}$ mice had less cardiac fibrosis and no increase in interstitial collagen deposition or impairment of systolic and diastolic function, maintaining heart function comparable to that of young mice. Cytokines are also involved in the role played by NETs in cardiac fibrosis. Previous research has demonstrated that low-density lipoprotein receptor-related protein 1 (LRP1) functions as an entogenic receptor for midkine (MK)[55,56], This pro-proliferative and pro-inflammatory cytokine [57] increases neutrophil adhesion and migration during acute inflammation via LRP1 interaction with β2 integrin [58–60] and initiates NETosis via interacting with MK. In mice with experimental autoimmune myocarditis (EAM), MK has the dual function of promoting polymorphonuclear neutrophil (PMN) recruitment and NET formation. The reduction in MK not only inhibits NETosis but also alleviates myocardial fibrosis and maintains cardiac systolic function [61].

The regulation of cardiac fibrosis involves several members of the chemokine family [62]. CXC chemokines with elicitin response (ELR) motifs may promote fibrosis by attracting active neutrophils and their subsequent NETs or by activating fibrotic monocytes. CXC Motif Chemokine Ligand 8 (CXCL8)/IL-8, a common human ELR-positive CXC chemokine that is also a powerful neutrophil chemoattractant, may activate the fibrotic action of neutrophils via the formation of NETs [63,64]. CXCL4/platelet factor-4 also has a role in cardiac remodeling [65]. In addition to myocardial fibrosis, NETs are involved in the development of thrombofibrosis and vascular fibrosis. Venous thrombosis, a disorder that can be lethal [66], can easily transform into chronic thrombosis, which is characterized as fibrosis mediated by neutrophil-mediated inflammation. Sharma et al. observed that thrombotic infection induces NETs, delays thrombolysis, and promotes fibrosis [67]. Whereas the genes linked to neutrophil inflammation were upregulated in the thrombi of mice infected with Staphylococcus aureus, they were downregulated significantly in mice treated with DNase1. Furthermore, NETs have been observed to induce monocytes to differentiate into fibroblasts in the thrombi of patients with chronic thromboembolic pulmonary hypertension (CTEPH), and TGFβ-mediated thrombus fibrosis has been demonstrated to be the downstream pathway of NET stimulation.

The link between NETs and TGF-β signaling can promote thrombotic persistence and fibrosis, resulting in severe consequences. Previous research has demonstrated a correlation between and NETosis and

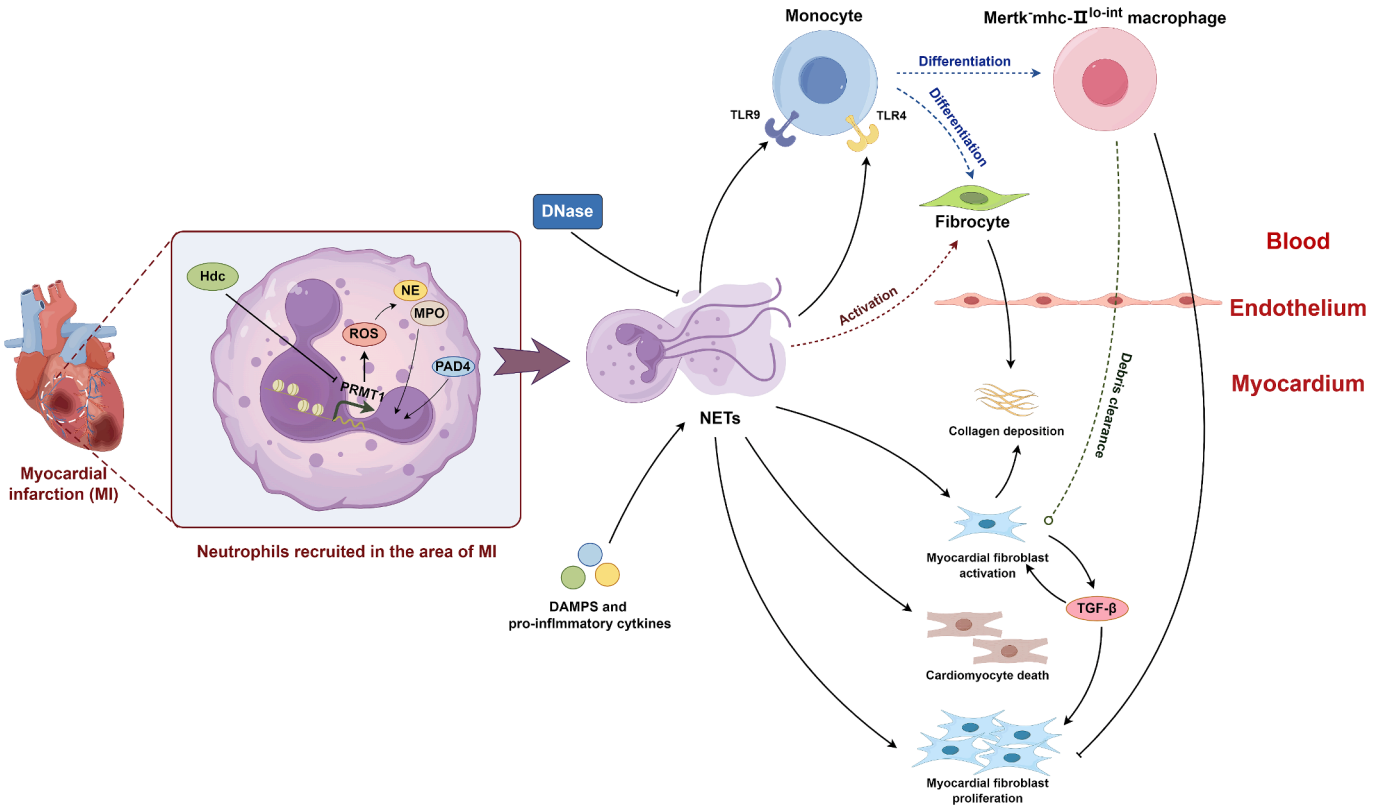

Fig. 3. The role of NETs in cardiac fibrosis post myocardial infarction (MI). Histidine decarboxylase (Hdc) deficiency enhances ROS-dependent NETosis, exacerbates cardiomyocyte death, and promotes myocardial fibrosis by relieving the inhibition of PRMT1 expression. Under MI-induced DAMPs or inflammatory stimulation, NETs drive the TLR9 or TLR4 pathway, promoting differentiation of monocytes into myeloid-epithelial-reproductive tyrosine kinase/major histocompatibility complex II (Mertk^{-}MHC-II^{lo-int}) macrophages or fibrocytes respectively, or directly activating fibrocytes, thereby contributing to the development of cardiac fibrosis. Additionally, damaged endothelial cells allow for the entry of NETs into the myocardium where they activate fibroblasts. Substances such as TGFβ secreted by these fibroblasts act on themselves or nearby fibroblasts to further synergistically upregulate cardiac fibrosis. By Figdraw.

atherosclerosis, which is characterized by an unregulated accumulation of collagen that causes arterial stenosis [68,69]. Chrysanthopoulou et al. discovered that TF-delivering thrombotic NETs may activate in human aortic endothelial cells (HAoECs) *in vitro*, triggering considerable cellular communication network factor 2 (CCN2) expression and endothelial collagen synthesis, resulting in atherosclerosis-related vascular fibrosis events [70]. The researchers further revealed that Ang II promoted *in vitro* NETosis through an ROS/PAD4 and autophagy-dependent pathway, indicating the importance of the Ang II/ROS/NET/TF axis in pathogenesis.

In conclusion, the relationship between NETs and cardiac fibrosis underscores the complex interplay between the immune system and chronic disease. Although the role of NETs in cardiac fibrosis remains an area of active research, accumulating evidence suggests that the development and progression of this debilitating disease is affected by dysregulation of the immune system, and in particular the activation of NETs. By further exploring this relationship, researchers may be able to identify new avenues for therapeutic intervention and more effective treatments for patients with cardiac fibrosis.

2.2. The respiratory system

2.2.1. Pulmonary fibrosis

Pulmonary fibrosis (PF) is a chronic, progressive respiratory disease with a poor prognosis that is often fatal. Studies have shown that the activation or proliferation of lung fibroblasts (LFs) plays a significant role in PF, and studies of agents used to induce PF have provided growing evidence that NETs have a role in PF development. Both Warheit-Niemi et al. [71] and Suzuki et al. [72] observed an increase in neutrophil count in the bronchoalveolar lavage fluid (BALF) of bleomycin-treated mice. Likewise, Warheit-Niemi et al. [71] found a significantly elevated level of extracellular DNA (exDNA) in the BALF of fibrotic mice with progressive phenotype and adverse disease outcomes, suggesting that neutrophils release more NETs under these conditions. Suzuki et al. [72] directly observed the appearance of NETs through fluorescence staining of lung tissue, indicating that NET formation is involved in PF development. In mice treated with benzyl butyl phthalate (BBP), Wang et al. found that BBP-induced neutrophil-released NETs promoted fibrosis of immortalized mouse lung epithelial 12 (MLE-12) cells in PF [73]. In addition, Chrysanthopoulou et al. demonstrated that NETs can induce the activation of LFs and their differentiation into myofibroblast (MF) phenotypes via inhalation of tobacco and magnesium silicate and administration of fibro-related drugs [74].

2.2.2. Idiopathic pulmonary fibrosis

Idiopathic pulmonary fibrosis (IPF) is a common progressive, irreversible, chronic lung disease characterized by collagen deposition in the lung interstitium. Current research indicates a potential role for neutrophils in the development of PF among individuals with IPF. Numerous clinical investigations have identified a link between neutrophil count and the advancement of IPF, particularly an increased number of neutrophils in the BALF of patients with IPF [71,75–77]. JP Lynch et al. reported that CXCL-8 promotes the development of pulmonary fibrosis by participating in the neutrophil chemotactic process [77]. They also observed that matrix metalloproteinase 8 (MMP-8) and MMP-9, two matrix metalloproteinases secreted by neutrophils, promote pulmonary fibrosis by inactivating IL-10 and activating TGF-β, respectively. Warheit-Niemi et al. observed dysregulation of neutrophil

survival and clearance in fibrotic mice, along with heightened release of elastase and NETs by neutrophils [71]. Although research has not yet established a direct connection between NETs and IPF, ample evidence indicates the role of NETs in the development of PF, suggesting a link between NETs and IPF.

2.2.3. Interstitial lung disease

Research indicates a link between NETs and PF in individuals with complications from interstitial lung disease (ILD) caused by autoimmune reactions. Observation of elevated levels of LL-37 in plasma, an element of NETs, and circulating cell-free NETs in ILD-associated autoimmune rheumatism coupled with reduced DNase activity indicate a connection between prolonged NET exposure and ILD onset. [78]. Within rheumatoid arthritis-associated interstitial lung disease (RA-ILD), plasma cell-produced anti-cyclic citrullinated peptide (CCP) antibodies induce the release of NETs, and NET-induced myofibroblast phenotypes in lung fibroblasts have been observed in ILD biopsy specimens [79].

In systemic lupus erythematosus-associated interstitial lung disease (SLE-ILD), the presence of hypoxia inducible factor-1α (HIF-1α) and endothelin-1 (ET-1) induce neutrophils to express regulated in development and DNA damage response 1 (REDD1), a stress-response protein. The REDD1/autophagy/NET axis is responsible for end-organ damage and fibrosis in SLE, with IL-17A in NETs playing a role in the fibrotic process in the dynamic inflammatory microenvironment of SLE [80]. During the process of anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis lung fibrosis (AAV-ILD), activated neutrophils infiltrate the interstitial perivascular space and release NETs that attract fibroblasts by damaging the endothelium. These NETs induce fibroblast differentiation and proliferation and collagen production through their nucleoid-associated proteins (NAPs), which are associated with NETs, and inhibit fibroblast apoptosis [81]. In polymyositis-associated interstitial lung diseases (PM-ILD), NETs have been shown to be involved in the proliferation and differentiation of lung fibroblasts (LFs) to myofibroblasts (MFs) via the TLR9-miR-7-Smad2 signaling pathway, thereby playing a role in accelerating ILD progression and promoting PF [82].

2.2.4. Cystic fibrosis

Characterized by alterations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, cystic fibrosis (CF), an autosomal recessive genetic disorder, can lead to the malfunction and harm of various bodily systems [83,84], with lung disease being the primary contributor for unfavorable outcomes [85,86]. The mutation of the cystic fibrosis transmembrane conductance regulator (CFTR) gene leads to a reduction in the activity of its encoded protein and an excessive rise in Na^+ and Cl^- levels in the secretory fluid of patients with CG, causing dehydration of secretions in the airway [87,88]. If the secretions are retained and blocked in the airway, they generate chronic obstruction, infection, and inflammation. An increasing number of studies have confirmed that the excessive formation of NETs by the infiltration of neutrophils in the airways of patients with CF promotes the thickening of alveolar mucus [89–92], impeding the clearance of airway mucus and allowing bacteria such as *Pseudomonas aeruginosa* to colonize [93,94]. This colonization in turn continues to promote neutrophil infiltration and NETosis, further increasing sputum viscosity [95]. Elastase, myeloperoxidase (MPO) and other NET components also play a role in sustaining intense inflammation and contributing to airway damage [96–99].

2.2.5. PF post COVID-19

NETs have also played an important role in the pathogenesis of PF post COVID-19. Neutrophils can release NETs to catch and eliminate SARS-CoV-2 after it has infected the human body. However, excessive and persistent NET release can cause ROS burst, leading to collateral lung damage [100–102]. Several studies have found that NET release is enhanced in severe COVID-19 [103–105]. Pandolfi et al. found that NETs in the BALF from patients with severe COVID-19 can induce epithelial-mesenchymal transformation (EMT) of lung epithelial cells [106]. In studies of ex-COVID-19 fibrosis induced by a chemical or infectious irritant, NETs and NAPs have been reported to facilitate the expression of genes for inflammation and fibrosis [72], or increase the quantity and activity of fibroblasts [81] (Fig. 4).

The means by which NETs cause PF remain unclear, but the prevailing opinion holds that NETs contribute to fibrosis via the TGF-β/Smad2/3 pathway [9,26] and function as upstream triggers of TGF-β [5], a classical pro-fibrosis factor. Several researchers have hypothesized that NAPs may play a mediating role in the development of fibrosis by modifying the composition of ECM; increasing the levels of collagen, osteopontin, and fibronectin; or directly stimulating the proliferation and differentiation of fibroblasts and inhibiting fibroblast apoptosis [81]. In addition, IL-17, which is expressed in NETs and can upregulate the expression of connective tissue growth factor (CCN2) and collagen synthesis to induce fibrotic response [74], is considered an important initiator of fibrosis [74].

2.3. The digestive system

Hepatic fibrosis, a necessary stage in the development of chronic liver disease to cirrhosis, is characterized by abnormal hyperplasia of connective tissue in the liver. Research groups have reported the accumulation of neutrophils in hepatic tissue promoting liver injury [107], including in non-alcoholic fatty liver disease (NAFLD), the predominant chronic liver disease. Fibrosis linked to hypercoagulability caused by the interaction of procoagulants and anticoagulants is one of the mechanisms underlying the clinical symptoms of NAFLD [108]. NETs are considered pro-coagulant substances that can cause the development of hypercoagulability by means of a surface supporting the activation of clotting factors [109,110].

NETs have been detected in the initial phase of liver damage caused by a methionine-choline-deficiency and high-fat (MCDHF) diet, but the extent of the damage can be attenuated by intraperitoneal injection of DNase. Stemming from membrane sphingolipid metabolism, sphingomyelin 1-phosphate (S1P) induces cell infiltration and activation via S1P receptors (S1PRs), playing a key role in controlling vital physiological and pathological activities. Zhao et al found that knockdown of S1PR improves liver inflammation and fibrosis through the suppression of NET formation [111]. In alcoholic hepatitis (AH), the breakdown of the intestinal barrier by alcohol results in excessive transfer of the bacterial metabolite lipopolysaccharide (LPS) to the liver, causing overproduction of NETs in the liver via the TLR4 of neutrophils. This process intensifies AH development, notably through fibrosis [112].

The development portal hypertension, the result of increased intrahepatic vascular resistance due to impaired hepatic sinus circulation and a primary cause of chronic hepatic congestion, is closely related to liver fibrosis [113]. Simonetto et al. demonstrated that sinus thrombosis contributes to chronic hepatic congestion in liver fibrosis in a mouse model with partial ligation of the inferior vena cava [114]. Chronic hepatic congestion mechanically stimulates liver sinusoidal endothelial cells (LSECs), a type of cell directly involved in increased hepatic resistance, to release angiocrine factors such as CXCL1, while promoting neutrophil recruitment at thrombosis sites to create NETs. Inhibiting formation of NETs correspondingly reduces the levels of liver fibrosis and portal hypertension, further underscoring the role of hepatic thrombosis in these conditions [115].

Additional research has pinpointed NETs as important mediators of thrombosis. NETs attach to red blood cells and platelets and their adhesion molecules, creating structures that trigger platelet activation and blood clotting, as well as promote both the endogenous and exogenous clotting pathways primarily by activating neutrophil serine proteinases. In a study of portal vein thrombosis (PVT), Xing et al. observed that NETs were more abundant in patients with PVT than those without

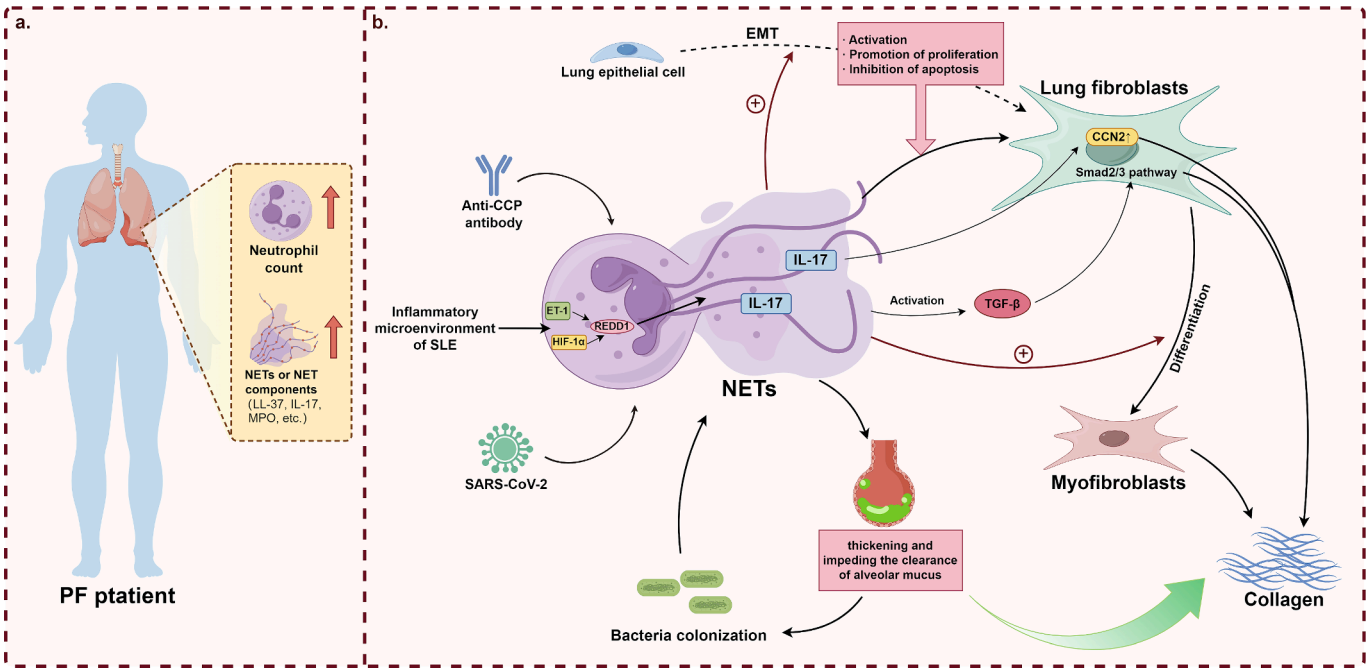

Fig. 4. The effects of neutrophils in pulmonary fibrosis (PF). A considerable body of evidence substantiates the proposition that NETs play a facilitative role in the pathogenesis of interstitial lung disease (ILD), cystic fibrosis (CF), and PF post to COVID-19 infection. A direct causal association between NETs and idiopathic pulmonary fibrosis (IPF) remains to be established. EMT, epithelial-mesenchymal transformation; ↑, up-regulated expression; ⊕, induction. By Figdraw.

PVT and that NET formation plays a role in the hypercoagulability of patients with PVT [116] (Fig. 5).

In cirrhosis, the final stage of liver fibrosis, the release of DAMPs by necrotic liver cells triggers cirrhosis of any etiology to cause localized inflammation, resulting in fibrosis and loss of liver tissue structure [117]. However, the role of NETs in cirrhosis and their link with fibrosis remains unclear. Zenlander et al. found an increase in plasma concentrations of the NET-forming MPO-DNA and citrullinated histone H3 (H3Cit) DNA in patients with cirrhosis and that their levels correlated with the extent of liver dysfunction [118]. Zermatten et al. observed an increase in NETs in the plasma of patients with cirrhosis, which is potentially linked to decreased liver clearance [119]. However, other researchers have yielded outcomes that challenge these observations. Among them, Agraz-Cibrian et al. revealed that neutrophils in

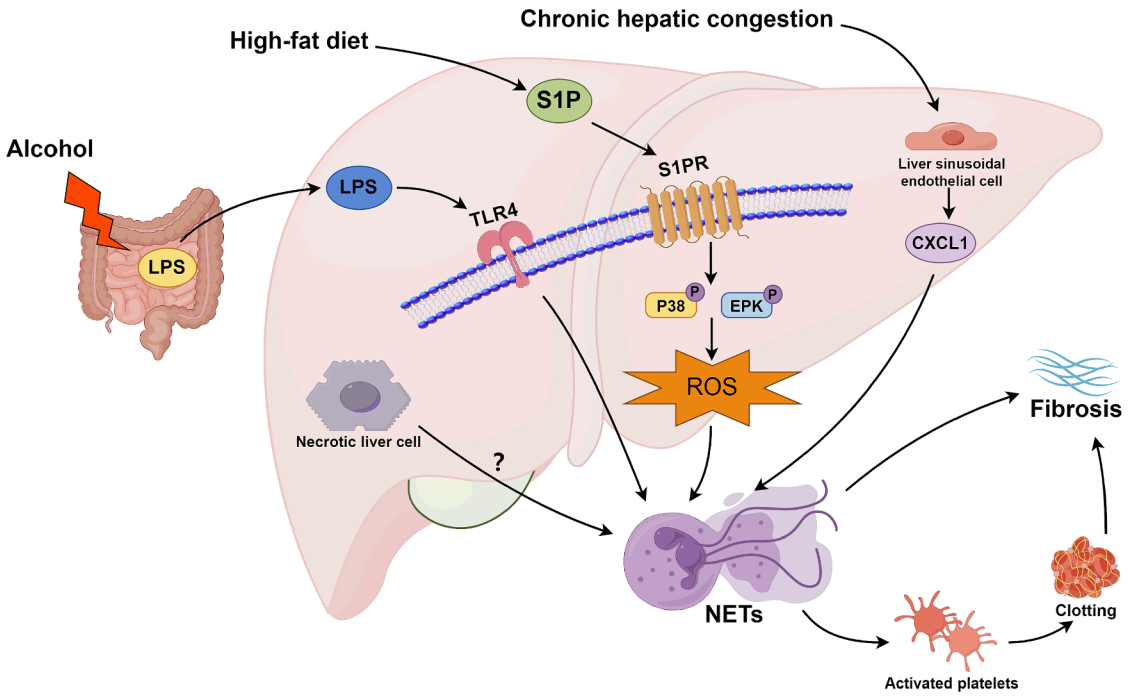

Fig. 5. The underlying mechanisms of NETs contributing to the pathogenesis of liver fibrosis. The roles that NETs play in promoting liver fibrosis in the context of non-alcoholic fatty liver disease (NAFLD), alcoholic hepatitis (AH), and chronic hepatic congestion attributable to portal hypertension have been demonstrated to some extent. Conclusive evidence establishing a direct causative link between NETs and the progression to cirrhosis remains elusive. LPS, Lipopolysaccharide; S1P, sphingomyelin 1-phosphat; S1PR, S1P receptor; EPK, Eukaryotic Protein Kinase; CXCL1, CXC Motif Chemokine Ligand 1; By Fidgraw.

patients with cirrhosis are deficient in ROS production, which is essential for NET activation, leading to a markedly diminished capacity for NET release [120]. More experimental evidence and clinical data that build on these findings are required to elucidate the role of NETs in patients with cirrhosis and its association with fibrosis.

2.4. The urinary system

Research indicates the potential role of NETs in the emergence and progression of renal fibrosis, a chronic, progressive renal disorder resulting from a range of progressive kidney diseases. Wang et al. found that unilateral ureteral obstruction (UUO) triggers the activation of gasdermin D (GSDMD) in neutrophils, leading to the creation of numerous NETs [121]. UUO also promotes macrophage-to-myofibroblast transition (MMT) through activation of the TGF-β1/Smad pathway, which is characterized by the expression of alpha-smooth muscle actin (α-SMA) in macrophages. In addition, the researchers demonstrated that caspase-11 is capable of cleaving GSDMD and is required for the development of post-UUO NETs and renal fibrosis. They found that removal of GSDMD in neutrophils markedly decreased the infiltration of inflammatory cells in the kidney and curtailed the formation of NETs and MMT, thereby preventing renal fibrosis.

Diabetic kidney disease (DKD) manifests through proteinuria, basement membrane thickening, mesangial area expansion, persistent renal fibrosis, and gradual renal deterioration. Within renal tissues affected by DKD, a higher number of neutrophils develop NETosis. Previous experiments have shown that a concentration of 25 mM glucose enhances the secretion of NETs and accelerates NETosis beyond a concentration of 5 mM glucose both *in vitro* and in patients with type 2 diabetes [122]. Moreover, the extent of NETosis has been found to be significantly increased in type 1 diabetes [123]. Liu et al. found an elevation in MK expression within the renal tissues of patients with DKD, with neutrophil proliferation correlating with MK concentration. MK has also been shown to significantly promote NETosis in hyperglycemically treated neutrophils. In a diabetic mouse model, MK promoted pathological damage and fibrosis of kidney tissue, whereas blocking MK through injection of MK antisense oligonucleotide decreased the formation of NETs [124].

Active SLE is marked by fibrosis of target organs, including the kidneys. The intensification of fibrosis in specific organs is mediated by TF and IL-17A in NETs released by neutrophils [125]. It is hypothesized that NETs play a role in disruption of capsule formation of glomerular crescents in patients with lupus nephritis,[80]. Several studies have shown that NET components trigger apoptosis of endothelial cells and degrade the basement membrane, leading to decreased glomerular blood flow and formation of a NETotic environment [126–128]. During hypoxia and renal injury, epithelial tubule cells further activate NETosis by releasing histones and thus forming a pro-inflammatory cycle that intensifies endothelial cell injury [128,129]. An overabundance of NETs may trigger vascular endothelial-cadherin proteolysis, resulting in loss of intercellular connections and vascular leakage and prompting endothelial-to-mesenchymal transition (EndMT), which fosters renal interstitial fibrosis by altering cell shape, polarity, motility, and collagen production via β-catenin signaling [129–131] (Fig. 6).

The accumulating evidence underscores the critical role that NETs

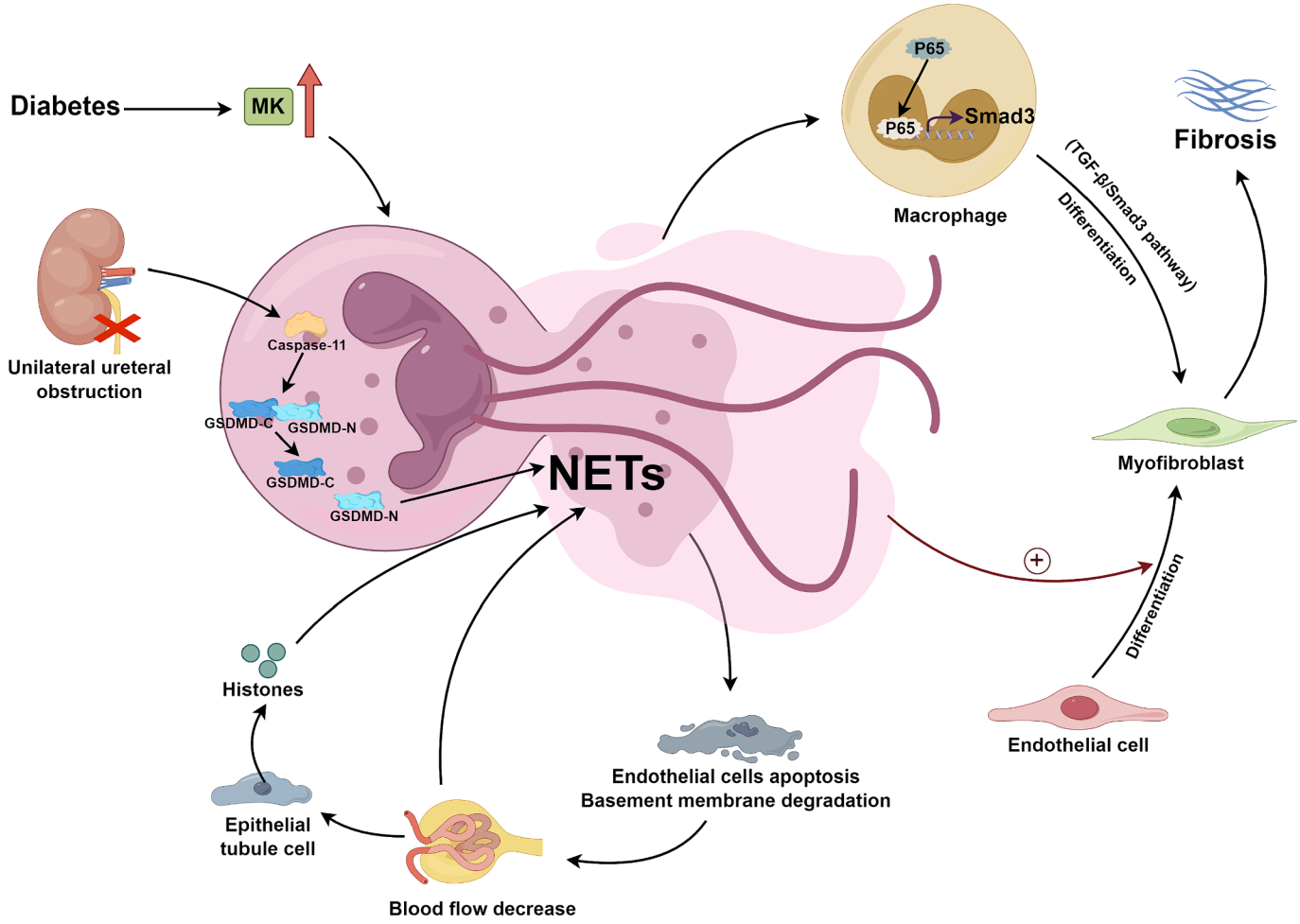

Fig. 6. Schematic model of NETs promoting renal fibrosis. GSDMD, gasdermin D; MK, midkine;↑, up-regulated expression; ⊕, induction. By Figdraw.

play across a spectrum of renal pathologies. The insights from these studies may pave the way for the development of novel therapeutic interventions. Future research should continue to elucidate the multifaceted roles of NETs in renal fibrosis, exploring both their pathological contributions and their potential as targets for innovative treatments.

2.5. The reproductive system

Although extensive research into the role of NETs in fibrotic disorders linked to the reproductive system is lacking, several researchers have discovered that NETs are capable of inducing ovarian fibrosis. The primary pathological changes in premature ovarian failure (POF) are reduced development of mature ovarian follicles and increased development of atretic follicles. Although the exact cause of POF remains unclear, several studies indicate that tissue fibrosis plays a significant role in its development and that excessive deposition of NETs may participate in the pathogenesis of POF by stimulating the release of cytokines to cause fibrosis. Observing that prolonged exposure to low-dose vitamin D (VD) significantly inhibited NET formation in tissues [132], Chen et al. proposed that VD could decrease the accumulation of ECM in ovarian tissue by preventing NET formation and downregulating the TGF-β1/α-SMA signaling pathway, thus decreasing ovarian fibrosis severity and enhancing ovarian functionality [133]. Despite these findings, knowledge of the role of NETs in fibrotic diseases linked to the reproductive system remains lacking, and more experimental and clinical research is needed to investigate the role of NETs in this context in the future.

2.6. The hematopoietic system

Recent studies have identified a potential role for NETs in the hematopoietic system. Chronic inflammation is considered the key process in the progression of early myeloproliferative neoplasms (MPNs) to obvious myelofibrosis. Studies into the role of NETs in the development of this disease are scarce, yet several investigations indicate that persistent inflammation in MPN partially stems from neutrophil apoptosis and survival imbalance, with the pro-inflammatory bone marrow microenvironment believed to support the formation of NETs [134]. The unstimulated neutrophils of patients with MPN have been found have a higher propensity to develop NETs compared with those from healthy controls. Using PMA and ionomycin for stimulation, Wolach et al. observed a higher frequency of NET formation by MPN neutrophils [135], and both Wang et al. and Münzer et al. found that the formation of NLR family pyrin domain containing 3 (NLRP3) inflammasome aids in the formation of NETs [136,137]. Zhou et al observed heightened expression of the inflammasome-related genes NLRP3, NF-κβ1, caspase recruitment domain family member 8 (CARD8), IL-1β, and IL-18 in bone marrow cells, which is associated with JAK2V617F mutation [138]. In accordance, recent discoveries have unveiled the function of JAK2V617F in triggering inflammation [139] (Fig. 7).

2.7. The skin and connective tissue system

Systemic sclerosis (SSc) is a rare autoimmune disease with a poor prognosis that is characterized by that development of progressive fibrosis. The pathogenesis of SSc is complex, encompassing the hardening and constriction of the impacted skin and affecting various organs, including the lungs, stomach, heart, and kidneys [140]. Impellizzieri et al. found that SSc is associated with neutrophil dysfunction, including elevated phosphorylation of STAT3 and STAT6, reduced expression of CD16 and CD62L, absence of chemokine receptors CXCR1 and CXCR2, diminished intracellular MPO post-activation, and less production of NETs, which they concluded to stem from prolonged activation of neutrophils by specific cytokines [141]. However, Didier et al. observed an increase in NETs produced by neutrophils in patients with SSc [142] and that these neutrophils were more likely to produce NETs in various

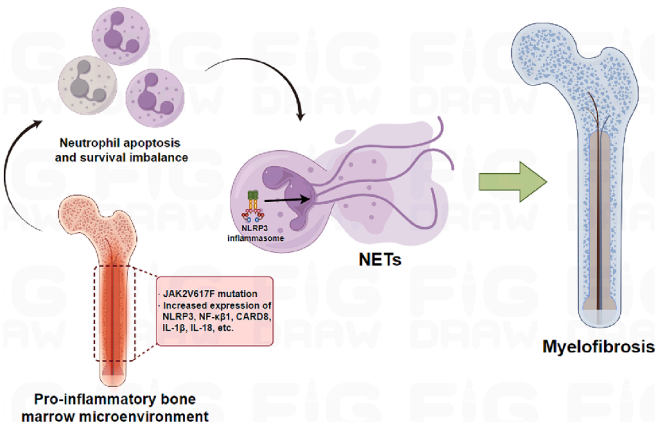

Fig. 7. The potential involvement of NETs in the pathological transition from myeloproliferative neoplasms (MPNs) to myelofibrosis. By Fidgraw.

subtypes of SSc, regardless of whether complications occurred, findings inconsistent with Impellizzieri et al.'s conclusion. In addition, Didier et al. observed that the increase in NET production was more significant and persistent in patients with severe vascular complications and less significant and persistent in patients with relatively mild complications (only fibrosis) or no complications [142]. Further research is essential to elucidate NET function in SSc and demonstrate the role of NETs and NETosis in SSc pathogenesis to pave the way for development of novel therapeutic strategies.

Scarring is commonly seen after trauma and surgery. Hyperplastic scarring is a type of pathological scarring that continues to grow and invade the surrounding healthy skin [143–145]. The prevailing view is that hyperplastic scars are characterized by an imbalance in fibroblast proliferation and apoptosis near the scarring site, infiltration of inflammatory cells, heightened collagen fiber expression, and substantial collagen accumulation [146–148]. In a recent study linking NETs to the emergence of hyperplastic scars, Shao et al. observed a notable rise in both neutrophil penetration and NETs in hypertrophic scars relative to normal scars. Moreover, they detected a higher propensity for these neutrophils to generate NETs transferred to fibroblasts through the TLR-9 receptor, leading to the activation of NF-κB in fibroblasts. This activation in turn boosts IL-6 secretion, which ultimately activates fibroblasts to secrete collagen and differentiate into myofibroblasts [149] (Fig. 8).

For scarring, especially hyperplastic scarring, the linkage to NETs offers a novel perspective on how immune responses can exacerbate fibrotic tissue formation, presenting potential targets for therapeutic intervention aimed at modulating NET activity.

2.8. The nervous system

Deep fibrotic scarring affects tissue function, including spinal tissue. The deep fibrotic scarring that results from spinal cord injury typically consists of fibrotic and glial scars. Spinal cord injury has recently been associated with NETs [150], and the critical function of minimizing fibrotic scar formation in aiding neurological recuperation has been gaining increasing recognition [151,152]. Feng et al. found that inhibiting the formation of NETs reduces both fibrotic and glial scars, thereby promoting the recovery of nerve function post spinal cord injury [153]. Epidural fibrosis after spinal surgery is a difficult clinical problem, with fibrotic tissue frequently causing complications such as low back pain and seriously affecting patient quality of life [154,155]. In a recent study, Jin et al. identified a potential link between NETs and the development of epidural fibrosis. In a laminectomy mouse model, Jin et al. found that NETs in wound tissue were stimulated by high mobility group protein B1 (HMGB1) and entered macrophages via the receptor for advanced glycation end product (RAGE), triggering the NF-κB/

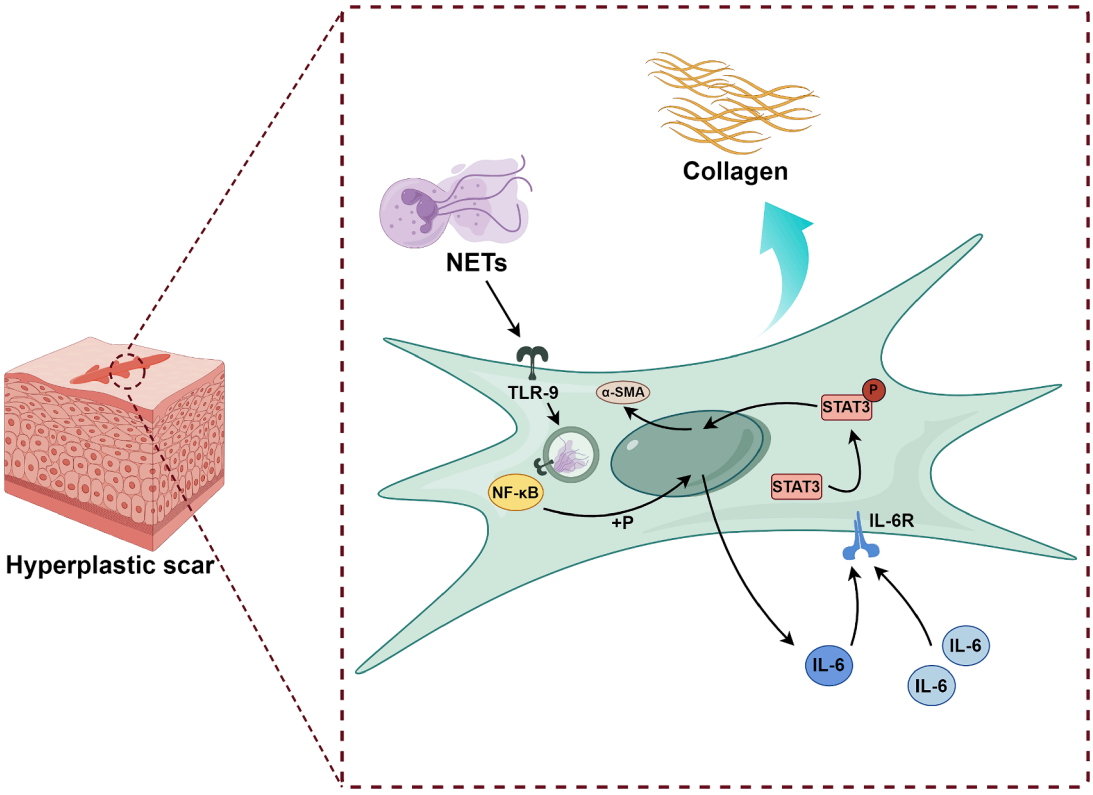

Fig. 8. The function of NETs in the development of hyperplastic scarring.Through the engagement of TLR-9, NETs are internalized by fibroblasts, a process which catalyzes the phosphorylation and subsequent nuclear translocation of NF-κB. This nuclear translocation of phosphorylated NF-κB (P-NF-κB) subsequently triggers the upregulation of IL-6 expression within fibroblasts. Elevated levels of IL-6, through both autocrine and paracrine signaling mechanisms, further stimulate fibroblasts to enhance the expression of α-SMA and escalate collagen synthesis.α-SMA, alpha-smooth muscle actin. By Fidgraw.

Smad3 signaling pathway and ultimately increasing α-SMA and fibronectin expression in macrophages. In contrast, they observed that administration of DNase decreased the severity of epidural fibrosis [156] (Fig. 9). Although studies of the role of NETs in scarring are lacking, current research indicates that NETs are potential targets for therapy. Further research into NETs could yield innovative strategies for treating scars and enhancing patient life quality.

3. The therapeutic potential of NETs in fibrotic diseases

The current treatments for fibrotic diseases are limited and the outcomes unsatisfying. With increasing research into the role of NETs and the pathogenesis of fibrotic diseases, it is becoming increasingly recognized that NETs may be targets for anti-fibrosis therapy. Given the partial identification of signaling pathways and molecular targets

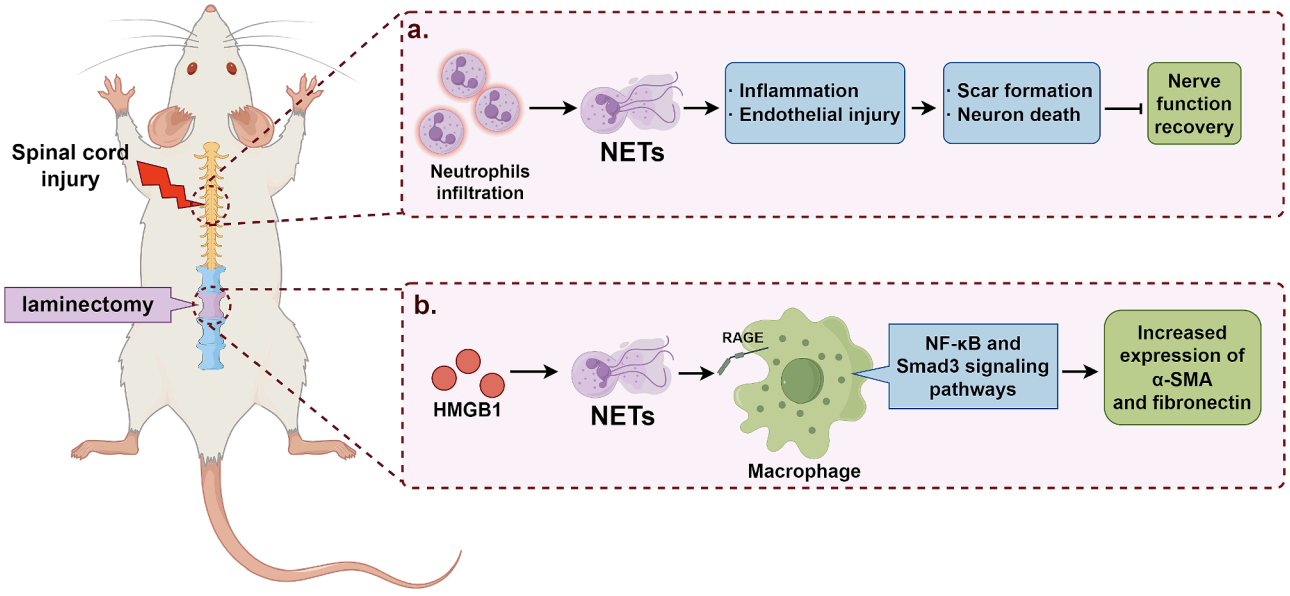

Fig. 9. Mechanism of role of NETs in spinal cord injury and epidural fibrosis in a mouse model. HMGB1, high mobility group protein B1; RAGE, receptor for advanced glycation end product. By Fidgraw.

associated with NETs, inhibiting or eliminating NETs may be an effective anti-fibrosis treatment.

The current treatment strategies targeting NETs primarily focus on inhibition of NET formation and promotion of NET degradation. Regarding the former, various potential inhibitors of NETs, such as ROS scavengers [102,157], PAD4 inhibitors [34–36,158], GSDMD inhibitors [121,159], and chemokine antibodies[160], are capable of attaining therapeutic benefits by interfering with NET formation or affecting neutrophil functionality. As the formation of thrombosis can lead to certain fibrotic conditions, anticoagulants such as heparin [118] and aspirin [118,161] may be able to disrupt NET formation and release or block the interaction between NETs and platelets to indirectly prevent NET-related thrombosis. By serving an anti-fibrotic function, immunosuppressants such as glucocorticoids may also decrease NET formation and decelerate fibrosis by modulating the immune response if administered early in disease development [162,163]. Among the strategies to counteract NETs, a focus has been placed on natural products known for their NET-inhibitory properties. Compounds such as Quercetin [164], Curcumin [165], Omega-3 fatty acids [166], Epigallocatechin Gallate [167], Vitamin D [133,166], and Ginsenosides [168] have been identified for their potential to modulate NET formation. These natural products, with their diverse bioactivities including anti-inflammatory, antioxidant, and immune-modulating effects, present promising avenues for the development of novel anti-fibrosis treatments.

Numerous research findings indicate that the application of DNase can eliminate NETs through DNA degradation, thus decelerating the fibrosis process. Nonetheless, other findings have suggested that treatment with DNase may enhance the release of NET degradation byproducts or leave other components of NETs in place, possibly leading to detrimental outcomes [169–172].

As present studies focusing on the interactions between NETs and fibrotic conditions primarily rely on fundamental experiments, the potential clinical application of their findings require verification. The potential of NETs as targets for anti-fibrosis therapy underscores an exciting frontier in medical research. Research is underway into novel medications and treatments, including inhibitors or activators aimed at protease secretion, autophagy, and apoptotic signaling in NETs. It is anticipated that these therapies will offer a broader range of options and opportunities for managing fibrosis.

4. Conclusions

Fibrosis is a common pathological process leading to multiple organ dysfunction and even life-threatening conditions. An increasing number of studies have been uncovering the crucial function of NETs in the emergence and progression of fibrosis, along with the anti-fibrotic therapeutic potential of inhibiting or clearing NETs. However, the journey from identifying potential NET inhibitors to implementing effective treatments is fraught with challenges. Technical obstacles, such as the precise measurement and control of NET activity in vivo, complicate the path to clinical application. Moreover, the ongoing investigation into the precise workings of NETs in fibrosis remains nascent, and more fundamental and clinical studies are needed to elucidate their functioning and regulation. Also required are more thorough assessment and refinement of the safety, effectiveness, and precision of medications and treatments targeting NETs to ensure that treatments do not inadvertently exacerbate the condition or introduce new health risks. Having significant importance both physiologically and pathologically, NETs, particularly their role in fibrosis, deserves greater attention and research, and further investigation and confirmation of their utility as potential targets for fibrosis therapy are of great necessity.

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Xiaojiao Wu: Writing – original draft, Project administration, Methodology, Investigation, Formal analysis. **Yang Yang:** Writing – review & editing, Project administration, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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